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MECHANISM OF STARFISH SPAWNING. II. SOME ASPECTS OF ACTION OF A NEURAL SUBSTANCE OBTAINED FROM RADIAL NERVE

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That starfish spawning is induced by injecting a water extract of radial nerves into the coelomic cavity has been well established in about twenty starfish species (Chaet and McConnaughy, 1959; Chaet, 1964a, 1966b, 1967; Noumura and Kanatani, 1962; Unger, 1962). The induced spawning is due to the action of a substance, a polypeptide (Kanatani and Noumura, 1962; Kanatani, 1967; Chaet, 1964b, 1966a, b, 1967), which has been called a "gamete-shedding substance." Efforts to elucidate the mechanism of starfish spawning have been continued in our laboratory in an attempt to know the mode of action of such a polypeptide, which is probably a product of neurosecretion. In a preceding paper of this series (Kanatani and Ohguri, 1966) it was shown that the quantitative distribution of the active substance seems to correspond to the quantity of nervous tissue present in a given part of the body; it occurs also in the tube feet, body wall and cardiac stomach and its quantity is uniform in various parts of the nerve ring-radial nerve system (per dry weight of lyophilized nerve material). It has been further demonstrated that the substance occurs in the coelomic fluid only when starfish are undergoing natural spawning.

The present report deals with the results of some *in vitro* as well as *in vivo* experiments carried out with respect to the action of the active substance obtained from radial nerves on discharge of eggs from the ovary. The effect on spawning of deficiency of bivalent cations in the sea water was also examined, since the preliminary experiments suggest that dissolution of an intercellular cementing substance among the oocytes is a prerequisite for starfish spawning. Further, the relation between the contraction of the ovary and the action of the nerve extract was investigated, since Chaet (1966a) has postulated that the shedding substance contains a "contraction factor." The effect of nerve extract on spawning when it is applied from outside the body was also examined, in order to verify the notion that this neural substance is a neurosecretory substance but not a kind of pheromone which acts among individuals. Some of the results presented here have already been reported in a brief preliminary form (Kanatani, 1964, 1967).

Materials and Methods

The material mainly used was the starfish, *Asterias amurensis*. *Asterina pectinifera* was also used in some experiments. The method for obtaining radial nerves was the same as that described in a preceding paper (Kanatani and Ohguri, 1966).

To make the nerve extract, lyophilized radial nerves were homogenized in a small amount of deionized water, and centrifuged at 27,000 *g* or 60,000 *g* for 1 hour. Sodium chloride solution was added to the supernatant to make an isotonic nerve extract (final concentration, 5/9 *M*), which served as the original extract. For the experiments, the original extract was diluted with sea water to appropriate concentrations. The concentration of nerve extract was expressed as the original weight of lyophilized nerve per milliliter of the test solution.

Salt-free nerve extract was prepared as follows. Lyophilized nerve was homogenized in deionized water (40 mg/ml) and centrifuged at 60,000 *g* for 50 minutes. The supernatant (5.8 ml) was gel-filtrated on a Sephadex G-25 column (2.5 × 94 cm) in deionized water. The fractions were assayed with ovarian fragments placed in 5 ml of sea water containing 0.01 ml of the fraction to be tested. The fractions found to be active were pooled and lyophilized. This sample, dissolved in a small amount of deionized water, served as the original salt-free nerve extract.

When nerve extract was to be injected into the coelomic cavity, sodium chloride solution was added to the supernatant of the nerve extract to bring its concentration to 5/9 *M*.

For *in vitro* assay, ovaries were isolated, thoroughly rinsed with sea water, and cut into pieces, 1 to 1.5 cm in length, which were used in the manner described in the preceding study (Kanatani and Ohguri, 1966).

To assay the nerve extract for its contraction-inducing ability, the method of Gaddum (1953) was adopted, using de Jalon's solution with 0.5 m*M* magnesium chloride. An isolated rat uterus, suspended in air, was connected with a kymographic apparatus and the degree of its contraction was recorded when a given test solution was applied to the surface of the uterus. The bath temperature was 31° C. The uterus used was obtained from a proestrus rat of the Wistar strain. Oxytocin solution (Syntocinon, Sandoz, Lot No. 64027; 5 I. U./ml) was diluted and used as the reference standard.

For the *in vitro* experiments, van't Hoff's artificial sea water was prepared by mixing isotonic stock solutions of the component salts. The pH of the artificial sea water was adjusted to 8.3 with 0.05% $NaHCO_3$ and a small amount of 0.1 *N* sodium hydroxide.

For histological study, ovarian fragments ligated at their cut surfaces were fixed in Zenker's (formol) solution and embedded in paraffin. Sections were cut at 5 μ, and stained with azocarmine G, anilin blue and orange G.

Results

Effect of nerve extract on spawning in vitro

Some experiments were conducted *in vitro* in order to determine the mode of action of the nerve extract on the ovary of *Asterias amurensis* with respect to spawning.

The distal part of an isolated ovary was immersed in sea water containing nerve extract (500 μg. lyophilized nerve/ml), and its proximal part in sea water (Fig. 1A). After 1 hour the ovary was thoroughly rinsed with sea water (Fig. 1B) and its gonadal wall was torn with fine forceps to make several small slits, proximally, centrally, and at the proximal part of the treated distal region.

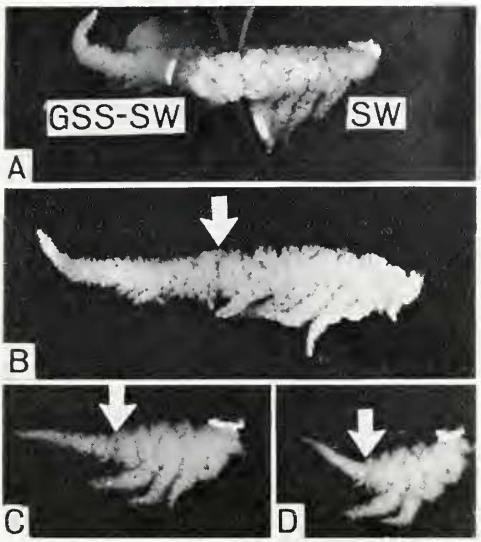

FIGURE. 1. Local treatment of a whole ovary with sea water containing nerve extract in *Asterias amurensis*; (A) an isolated ovary partially exposed to nerve extract and sea water; GSS-SW, sea water containing nerve extract; SW, sea water; (B) same ovary rinsed with sea water immediately after such treatment for 1 hour; arrow, boundary between treated and untreated regions; (C) Same ovary after discharge of eggs from treated portion; (D) same ovary after additional treatment with potassium chloride; B, C, D, same magnification.

Eggs were released immediately from these openings, but discharge of eggs from the regions not treated with nerve extract soon ceased. On the contrary, the shedding of eggs from the slits made in the portion treated with nerve extract was intense and continued so that the treated portion was clearly distinguishable from the nontreated portion. Figure 1C shows such an ovary 50 minutes after the slits were made. When these ovaries were again transferred to sea water containing potassium chloride at a high concentration (sea water 300 ml + 5/9 *M* KCl 60 ml), eggs were again discharged from the slits. The degree of discharge from the treated portion was very intense in contrast to the nontreated portion. Figure 1D shows the ovary 10 minutes after such treatment with potassium chloride.

Microscopic observation of the released eggs at the time of discharge revealed that those from the portion treated with nerve extract were surrounded only by jelly, whereas, those from the nontreated portion had follicles. Also, breakdown of the germinal vesicles was seen only in oocytes obtained from the treated portion. This was confirmed in another experiment in which ligated ovarian fragments were treated with nerve extract for 1 hour. The eggs within such fragments lacked follicles as well as germinal vesicles, and scattered freely when the ovary was torn, whereas eggs within the control (nontreated) fragments had both follicles and germinal vesicles, and tended to adhere to each other. Histological examination made on the ligated ovarian fragments immersed in sea water containing nerve extract (500 μg/ml) for 1 hour clearly confirmed these observations made on living material. As shown in Figure 2, oocytes within the treated ovarian frag-

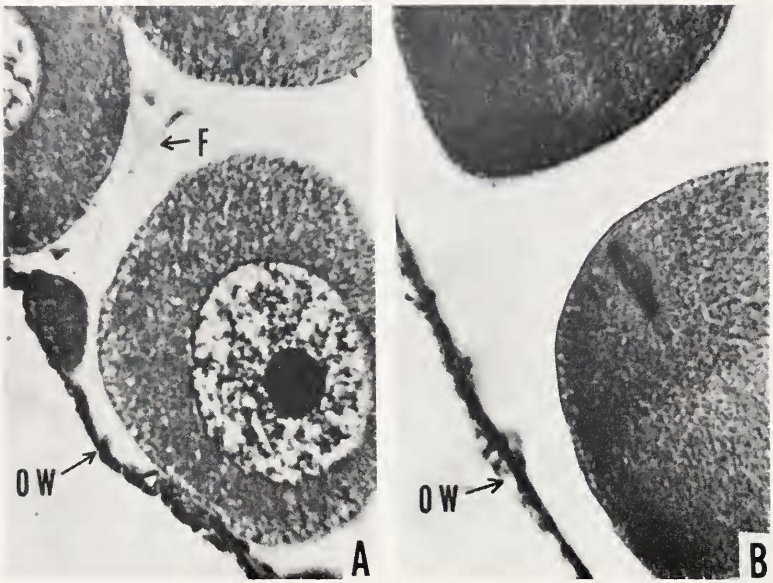

FIGURE 2. Maturation of oocytes within ovary brought about by nerve extract. (A) Section of ovarian fragment immersed for 1 hour in sea water (control). OW, ovarian wall; F, follicle. (B) Section of ovarian fragment immersed in sea water containing nerve extract for 1 hour. Note that oocytes are detached from the ovarian wall and undergoing first maturation division. Follicles surrounding oocytes disappear.

ments were detached from the ovarian wall and were undergoing the first maturation division, whereas oocytes in control fragments kept in sea water alone for the same period were attached to the ovarian wall, and between them a follicular envelop was observed. Germinal vesicles of the control oocytes remained intact.

These experiments showed that although contraction of the ovarian wall seemed to be essential for expelling the eggs from the ovary, there existed a marked difference between the portions treated with nerve extract and the controls with respect to the readiness with which eggs were released.

Further, when an ovarian alveolus was isolated from an ovary and its wall slit by fine forceps in sea water, the alevolar wall contracted suddenly, discharging a mass of eggs from the slit, and turning inside-out because of the presence of eggs adhering to its inner surface and to each other (Fig. 3). Most of the eggs in isolated alveoli treated with nerve extract were freely discharged after a certain period even without a slit being made.

From these results, it is concluded that the action of the neural substance results in freeing the eggs from adherence to each other and to the inner surface of the gonadal wall, by dissolving or rupturing the follicles.

Spawning of ovarian fragments in sea water lacking divalent cations

Since bivalent cations such as calcium and magnesium are well known to stablize intercellular cementing substances, the effects of deficiency of such divalent

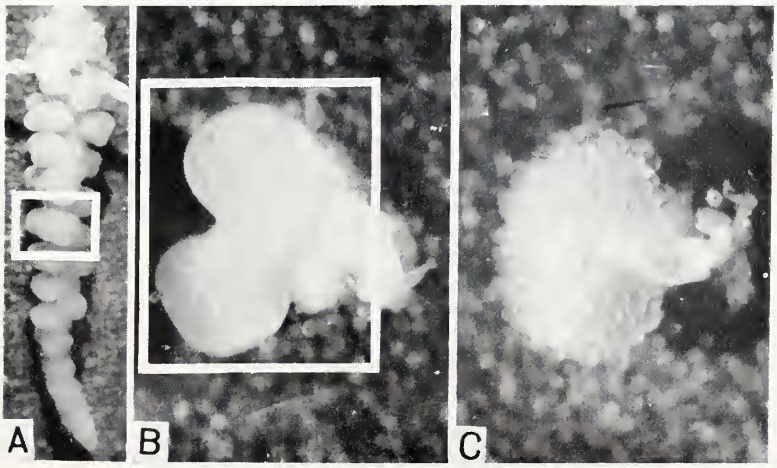

FIGURE 3. Adherence of eggs to each other and to the inner surface of the gonadal wall. (A) Ligated ovarian fragment of *Asterias amurensis*, white quadrangle shows an ovarian alveolus; (B) isolated ovarian alveolus; (C) same alveolus turns inside-out after its wall is slit. Note adherence of eggs to each other.

cations on spawning were next investigated. Isolated ovaries were transferred to magnesium-free sea water and rinsed three times with renewal of the medium, for five minutes each time. Small ovarian fragments were then removed and kept in sea water containing magnesium at various concentrations. Within half an hour the ovarian fragments immersed in media containing magnesium at lower concentrations began to shed eggs (Fig. 4). The critical concentration of magnesium at which spawning occurred was usually 3×10^{-3} *M* and the degree of spawning increased as the concentration of magnesium decreased. The intervals preceding the discharge of eggs and amounts of discharged eggs varied according to the condition of the gonad in the breeding season in the same way as in the nerve extract experiment. Later in the breeding season the ovary was usually more sensitive to spawning inducers.

The effect of lack of calcium on spawning was next studied. Ovaries were isolated and rinsed twice with calcium-free sea water before being cut into small pieces. The fragments were further washed five times (1 minute each) with fresh calcium-free sea water; a total of about ten minutes was needed for preparation. Ovarian fragments kept in calcium-free sea water failed to discharge eggs in contrast to those treated with magnesium-free sea water. Intensive spawning, however, suddenly occurred when these fragments treated with calcium-free sea water for a prolonged period were returned to sea water. As shown in Table I, a minimum treatment with calcium-free sea water of 45 minutes was required to induce copious spawning.

When ovarian fragments treated with calcium-free sea water were transferred to sea water containing calcium at various concentrations, the discharge of eggs became more intense as the concentration of calcium ions rose. Table II shows a representative result of such experiments. The concentration of calcium required for significant spawning was above 10^{-3} *M* in this case.

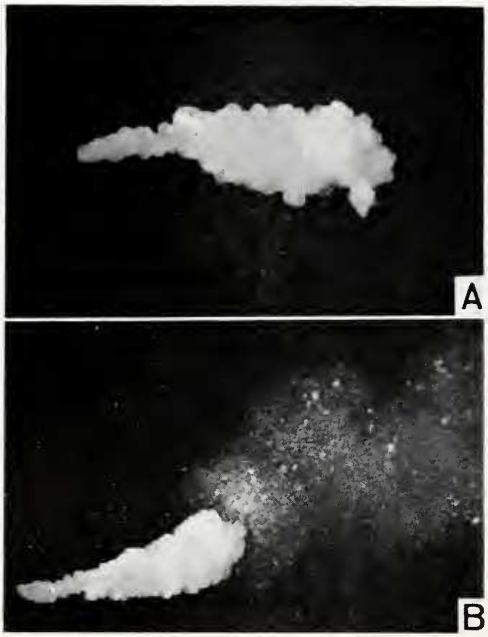

FIGURE 4. Spawning of ovarian fragment treated with magnesium-free sea water; (A) ovarian fragment before treatment with magnesium-free sea water; (B) same fragment discharging eggs in magnesium-free sea water.

In order to determine whether or not the effect of calcium deficiency is reversible, ovarian fragments, ligated to prevent discharge of eggs, were kept in calcium-free sea water for 50 minutes and then placed in sea water for appropriate periods up to 90 minutes. When cut at the ligated portion such fragments began to shed

TABLE I

Induction of spawning in ovarian fragments of Asterias amurensis *when they were kept in calcium-free sea water for various periods and then transferred to sea water*

Duration of treatment with calcium-free sea water (minutes)	Interval preceding discharge of eggs after transfer to sea water	Degree of spawning 30 minutes after transfer to sea water
10	(no spawning)	−*
15	(no spawning)	−
20	4–5 minutes	±
25	1–2 minutes	+
30	4–5 seconds	+
35	2–3 seconds	++
40	2–3 seconds	++
45	at once	+++
50	at once	+++
60	at once	+++

* −: no spawning; ±: very small amount of eggs; +: small amount of eggs; ++: intermediate amount of eggs; +++: large amount of eggs (ovarian fragments practically empty).

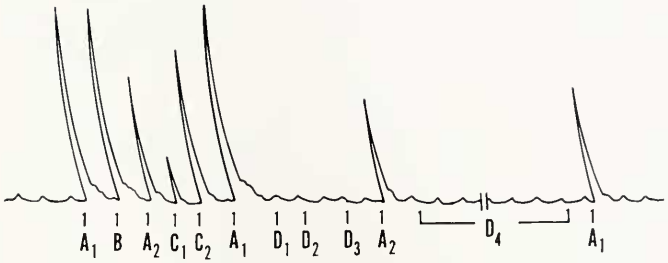

FIGURE 5. Contraction-inducing effects of crude and gel-filtrated extracts of starfish radial nerve on superfused rat uterus; tracings of original kymographic recordings: A_1, oxtocin (50 μU); A_2, oxtocin (25 μU); B, crude nerve extract of *Asterina pectinifera* (100 μg of dry nerve); C_1, crude nerve extract of *Asterias amurensis* (100 μg of dry nerve); C_2, same (200 μg of dry nerve); D_1, nerve extract of *Asterias amurensis* gel-filtrated on Sephadex G-25 (750 μg of dry nerve); D_2, same (360 μg of dry nerve); D_3, same (75 μg of dry nerve); D_4, same (750 μg/ml solution was perfused for 1 hour).

eggs at once. The degree of spawning was intense so that the fragments were practically emptied. Since the follicles were no longer observed around the eggs, this irreversibility of the effect of calcium deficiency seemed to be due to the fact that the subsequent addition of calcium failed to repair the broken follicles which had disintegrated under the previous condition of calcium lack. In the absence of calcium, ovarian fragments failed to spawn even in the presence of salt-free nerve extract (about 25 μg or 100 μg lyophilized nerve/ml), although breakdown

TABLE 11

Induction of spawning in ovarian fragments of Asterias amurensis *when they were treated with calcium-free sea water and then transferred to sea water containing calcium at various concentrations*

Concentration of calcium in test sea water	Duration of treatment with calcium-free sea water (minutes)				
	15	30	45	60	90
3×10^{-2} *M*	4–5 minutes* ±**	4–5 seconds* ++**	at once* +++**	at once* +++**	at once* +++**
10^{-2} *M*	—	4–5 seconds +	at once +++	at once +++	at once +++
3×10^{-3} *M*	—	20 seconds ±	at once ++	at once +++	2–3 seconds +++
10^{-3} *M*	—	1–2 minutes ±	6-7 seconds ++	6–7 seconds ++	2–3 seconds +++
3×10^{-4} *M*	—	—	3–4 minutes +	20 seconds +	20 seconds +
10^{-4} *M*	—	—	—	—	—

* Interval preceding discharge of eggs after transfer to medium containing calcium.
** Degree of spawning; see Table I.

of the germinal vesicles occurred within the fragments after 30 minutes. These fragments began to spawn immediately after addition of calcium.

Relation between contraction of ovary and nerve extract

It is evident that the contraction of the ovarian wall is essential for starfish spawning, in order to expel the eggs from the ovary. This contraction of the ovarian wall seems to be due to its muscular component. Although muscle cells were histologically difficult to recognize in the wall of the distended ovary during the breeding season, contracting muscle cells were clearly observed in histological preparations of the wall of spent ovarian fragments after treatment with nerve extract.

Some experiments were conducted to investigate (1) the effect of known contraction-inducing agents such as potassium chloride and acetylcholine chloride on the spawning of *Asterias amurensis;* (2) the contraction-inducing ability of nerve extract as checked by the superfusion method using rat uterus, a common pharmacological test, and (3) the effect of contraction-inducing agents and nerve extract on the contraction of the isolated gonadal wall of starfish.

When 5 ml of 5/9 *M* potassium chloride were injected into the coelomic cavities of *Asterias* (about 300 g in body weight), small amounts of sperm or eggs were discharged from the gonopores within a few minutes in some starfish. However, this release of gametes soon ceased. Injection of 5 ml of sea water containing 5×10^{-3} *M* acetylcholine chloride had the same effect. Addition of eserine at low concentrations to acetylcholine solution did not alter the results.

In vitro experiments, in which either 10 ml of sea water containing 5 ml of 5/9 *M* potassium chloride, or acetylcholine chloride in sea water (10^{-3} *M* final concentration) were used, showed that a small amount of eggs was immediately discharged from the cut surface, but the discharge soon ceased. When electric stimulation (20–40 volts, alternating current, for 10 seconds) was applied to a small ovarian fragment in sea water by placing it between two electrodes 2 cm apart, the reaction of the fragment was similar to that seen on treatment with potassium chloride or acetylcholine chloride. Under a dissection microscope, the eggs within the ovary were observed to elongate along the long axis of the ovarian alveoli when treated with these contraction-inducing agents suggesting that the ovarian wall was under tension. On the other hand, ovarian fragments taken from the same female and treated with nerve extract spawned copiously until they were practically empty.

It should be noted that the induction of this small-scale spawning by contraction-inducing agents such as potassium chloride and acetylcholine depended much upon the state of gonads. Some ovarian fragments, whose sister fragments spawned heavily with nerve extract, failed to respond to such agents, while others discharged eggs to a certain degree. Testis fragments usually responded well to these treatments and shed a cylindrical mass of sperm from the cut surface, suggesting that the gametes could be expelled more readily from the testis than from the ovary.

The effect of starfish nerve extract on muscle tissue was examined with superfused rat uterine muscle isolated from a proestrus rat (Gaddum, 1953), using synthetic oxytocin as the reference standard. Representative kymographic record-

ings show the contractability of the uterine preparation induced by oxytocin (Fig. 5A), and by crude nerve extracts of *Asterina pectinifera* (Fig. 5B) and *Asterias amurensis* (Fig. 5C). However when these crude extracts were gel-filtrated on a Sephadex G-25 column in order to remove possible contaminating substances of small molecular weight, the contraction-inducing ability was lost; contraction was no longer observed with various dosages such as 0.1 ml of 750 to 7,500 μg of lyophilized nerve of *Asterias* per ml. (Fig. $5D_{1-3}$). Finally, no contraction of the uterus was observed (Fig. $5D_4$), when 50 ml of the gel-filtrated nerve extract (750 μg lyophilized nerve per ml) was superfused for 1 hour, although this extract was very effective in inducing spawning, causing isolated ovaries to begin shedding their eggs within 20 minutes. The same uterine preparation contracted strongly on addition of 0.1 ml of 50 μU oxytocin even after these prolonged experiments (Fig. 5, last A peak). It is therefore concluded that the neural substance responsibile for spawning by itself was not the agent which caused contraction of the rat uterine muscle. The contraction observed following treatment with the crude nerve extract seems to be ascribable to some factor(s), contained in the nerve extract, other than the so-called shedding substance.

In order to compare the effffect of potassium chloride and acetylcholine chloride (Kanatani, 1967) on the contraction of the ovarian wall with that of the shedding substance, some experiments were conducted using ovary of *Asterias*. Kymographic recordings showed that strong contraction of isolated ovarian wall preparations was induced by both potassium chloride and acetylcholine chloride, and that the capacity of the gel-filtrated nerve extract to cause contraction of the ovarian wall was, if any, very slight, suggesting that the shedding substance acts on the ovary to induce spawning by some way other than by causing the ovary to contract.

Effect of treatment with hyaluronidase and trypsin on spawning

Some experiments were conducted to test whether treatment with hyaluronidase or trypsin would cause induction of spawning.

Injecting either 0.5 ml of sea water containing 0.5% hyaluronidase (NBC, 300 U/mg) or 0.2 ml of sea water containing 0.1% trypsin (Sigma, 10,000 U/mg) into the isolated ovarian fragments (*ca.* 3 cm in length) of *Asterias amurensis* failed to induce discharge of eggs. Ovarian fragments immersed in these solutions also failed to spawn. Masses of eggs adhering to the cut surfaces did not show any sign of dispersion. These results suggest that the intercellular cementing substance between oocytes resists the action of these enzymes.

Effect of nerve extract on spawning when applied from outside of the body

The neural substance was found in the coelomic fluid only when the starfish were undergoing natural spawning (Kanatani and Ohguri, 1966). Some experiments were carried out with *Asterias amurensis* to determine whether or not the active substance is released from the radial nerves *into the surrounding sea water* and then taken by the same or a neighboring animal to stimulate the release of gametes, as postulated by Chaet (1966b; 1967, pp. 16–17).

A total of sixty starfish (50–110 g in body weight) were kept separately for 4–8 hours in 500 ml of sea water containing nerve extract in various concentra-

TABLE III

Effect of sea water containing nerve extract on spawning when applied from outside of the body in Asterias amurensis

Date of experiment	Concentration of nerve extract	Period of treatment (hours)	No. of animals used	No. of animals spawned	No. of animals* spawned after injection with nerve extract
March 24	200 μg/ml	4	10 (♂7, ♀3)	0	8** (♂5, ♀3)
	100 μg/ml	4	10 (♂3, ♀7)	0	10 (♂3, ♀7)
	0 (control)	4	10 (♂3, ♀7)	0	10 (♂3, ♀7)
April 21	50 μg/ml	4.5	10 (♂1, ♀8, ?1)	0	5** (♂1, ♀4)
	25 μg/ml	4.5	10 (♂3, ♀7)	0	6** (♂2, ♀4)
April 23	200 μg/ml	8	10 (♂5, ♀5)	1	9*** (♂5, ♀4)
	100 μg/ml	8	10 (♂4, ♀6)	1	9*** (♂4, ♀5)
	0 (control)	8	15 (♂6, ♀9)	2 (♂1, ♀1)	13*** (♂5, ♀8)

* After treatment with the test solution, 1 ml of nerve extract (20–100 μg/ml) was injected into the coelomic cavity.

** Gonads poorly developed, or had already spawned, in animals which did not spawn on injection with nerve extract.

*** No injection was performed with animals which had already discharged their gametes.

tions (25–200 μg of lyophilized nerve per ml). Another twenty-five starfish kept in sea water alone served as controls. During the 8-hour treatment, the sea water was aerated throughout the experiment. After such treatment 1 or 2 ml of the nerve extract (20–100 μg/ml; the highest dosage was 100 μg/animal) was injected into the coelomic cavity in order to check the spawning capacity. The results are shown in Table III. Forty animals, both males and females, kept in sea water containing nerve extract (25–200 μg/ml) for 4 to 4.5 hours on March 24 and April 21 failed to spawn. However, when these animals were washed, transferred to fresh sea water, and injected with the nerve extract, they discharged eggs or sperm within 1 hour. Animals which did not spawn after injection were found on autopsy to have very small gonads. Although two of the 20 animals kept in sea water containing nerve extract (100–200 μg of dry nerve per ml) for 8 hours on April 23 spawned, two of the 15 control animals also discharged gametes. On the same day, natural spawning was observed in several starfish kept in other aquaria, suggesting that spawning in this case was not due to the action of the applied nerve extract. Both males and females which had failed to spawn in sea water containing nerve extract discharged sperm or eggs within 1 hour after injection of nerve extract (100 μg of dry nerve per animal).

In the final experiment of this series, the arms of starfish were slit with scissors for about 2 cm along the ambulacral plate, in order to introduce the external medium into the arm. In these experiments, six (3 males and 3 females) of the 7 starfish kept in sea water containing nerve extract (100 μg/ml) discharged their gametes within 2 hours. Further, four (2 males and 2 females) of the six exposed to 50 μg/ml and four (2 males and 2 females) of the five exposed to 25 μg/ml discharged their gametes.

From these experiments it is clear that the neural substance responsible for spawning has no effect when it is applied from the outside of the starfish body.

Discussion

Starfish spawning, as induced by injecting the nerve extract into the coelomic cavity or by treating isolated ovarian fragments with sea water containing nerve extract, is explained as follows. The polypeptide hormone contained in the radial nerve (Kanatani and Noumura, 1962; Kanatani, 1967, 1969; Chaet, 1967) is liberated so that it reaches the coelomic cavity, and there directly acts on the ovary from its surface to bring about dissolution of an intercellular cementing substance between the follicle cells and the ovarian wall, making the eggs within the ovary freely movable. Under these conditions the ovarian wall, whose contractability seems to be ascribable to its muscular component (Chaet, 1966a; Kanatani, 1967; Schuetz and Biggers, 1968), can contract actively and force out the individual eggs, thus bringing about spawning. The active substance itself does not seem to be a direct cause of the contraction of the ovarian wall, as will be discussed below.

A contrary opinion has been expressed by Chaet (1966ab), who believes that this substance causes the ovarian wall to contract actively, thus expelling the eggs. If the nerve extract brings about spawning by simply causing muscular contraction, spawning should also be induced by treatments which cause contraction of the gonadal wall. However, as shown in the present study, potassium chloride, acetylcholine chloride and electric stimulus all fail to induce significant spawning. In the case of sea urchins, application of these contraction-inducing agents is very successful in inducing spawning (Iida, 1942; Iwata, 1950; Harvey, 1952, 1953; Iwata and Fukase, 1964ab). Since the histological constituents of the ovarian walls in sea urchins and starfish are almost identical (Hamann, 1885; Kawaguti, 1965; Kanatani, unpublished data), and since the isolated ovarian wall of starfish itself contracts vigorously on treatment with potassium chloride and acetylcholine (Kanatani, 1967), the failure of contraction-inducing agents to induce spawning in starfish should be ascribed to some other cause. Observation of isolated ovarian alveoli in the present study suggests that the masses of eggs adhering to each other or to the inner surface of the gonadal wall obstruct the release of individual eggs, so that they resist the contraction of the ovarian wall. Sea urchin oocytes, on the other hand, mature within the ovary and are already free from the follicles long before spawning takes place (Fuji, 1960). This condition of the ovary seems to explain why the spawning of sea urchins can be readily induced by some contraction-inducing agents.

Experiments to test the contraction-inducing activity of starfish nerve extract, with rat uterus as well as with starfish ovarian wall, revealed that crude nerve extract has some effect in causing muscle tissue to contract. However, when the

nerve extract was partially purified by gel-filtration in order to remove possible contraction-inducing contaminants such as acetylcholine (Unger, 1962), it no longer showed such activity. It is therefore concluded that the ability of the active peptide to cause contraction of the ovarian wall is, if present at all, very slight, suggesting that its primary and main action is to dissolve the cementing substance surrounding the eggs. It is to be noted that our previous results (Kanatani, 1964), obtained in an experiment measuring changes in pressure within the ovarian fragments, should be re-examined since the experiment used crude nerve extracts without gel-filtration, and it is possible that contraction-inducing agents contaminating the extracts may affect pressure change.

Chaet's observation (1966a, p. 50) that the ovary began to contract in the presence of nerve extract several minutes before eggs were extruded through the gonopore (about 25 minutes after the treatment began) can be explained in another way; during this period of treatment the extract acts on the cementing substance within the ovary, to breakdown the follicles and to set free the eggs, so that individual eggs can be displaced within the distended ovary (whose wall is under considerable tension). As a result of this change, the surface area of the ovarian wall decreases, causing the ovary to decrease in length. A pronounced decrease in the length of ovary is seen after the oocytes have been discharged. Therefore, decrease in ovarian length after treatment with sea water containing varying concentrations of calcium and a constant amount of gel-filtrated nerve extract (Schuetz and Biggers, 1968) does not seem to contradict the present notion. Furthermore, in Chaet's experiment (1966a), the question arises as to why such a long period as 25 minutes elapses between the onset of treatment and the commencement of contraction. Since contraction of the ovarian wall does not require such a long latent period, this period is thought to represent the time needed for breakdown of follicles in the ovary. In our experience, potassium chloride or acetylcholine causes immediate contraction of the ovarian wall (Kanatani, 1967).

Although there is no doubt that contraction of the ovarian wall is a direct cause of the release of individual oocytes which have been liberated from binding to each other and to the gonadal wall, as a result of the breakdown of follicles under the influence of hormonal peptide, there is as yet no direct evidence available to explain how this contraction is realized. Although simple tearing of the ovarian alveolus causes the strong contraction of its wall, the effect of mechanical stimulation is not excluded in this case. That the isolated hormone peptide can induce spawning of ovarian fragments (Kanatani, unpublished data) seems to exclude the possibility that some unknown contraction-inducing substance, which might be released simultaneously from the nerve at the time of release of the hormonal peptide, is required to bring about spawning. What directly causes the active contraction of the ovarian wall remains unsolved; induction of spawning by keeping the ovarian fragments in magnesium-free sea water or by transferring them from calcium-free sea water to normal sea water (Kanatani, 1964, 1967; Schuetz and Biggers, 1968) suggests that no specific contraction-inducing agent is required.

The occurrence of spawning in ovarian fragments immersed in magnesium-free sea water without nerve extract, or in sea water lacking both calcium and magnesium without nerve extract (Kanatani, 1964) suggests, besides the dissolution of cementing substance, that magnesium may inhibit the discharge of oocytes;

this is in accord with the statement of Schuetz and Biggers (1968). According to Iwata and Fukase (1964b) magnesium anaesthetizes the neuro-muscular junction, and application of excess magnesium inhibits the spawning of sea urchins induced by acetylcholine. In calcium-free sea water without nerve extract ovarian fragments do not discharge their eggs. In this case, the presence of magnesium ions may be involved in the failure of spawning. The induction of spawning after adding calcium to calcium-free sea water might be a problem of the ionic balance between calcium and magnesium. On the other hand, although calcium ions are believed to play an important role in starfish spawning (Mecklenburg and Chaet, 1964; Kanatani, 1967; Schuetz and Biggers, 1968) ovarian fragments undergo discharge of eggs when they are kept in sea water lacking both calcium and magnesium ions (Kanatani, 1964; 1967). Some technical difficulty, always encountered in experiments using isolated whole ovary or fragments to remove the target ions exclusively, makes it impossible to draw any definite conclusion as to the effect of a given ion. At present, the exact effect of various ions on starfish spawning is far from clear. We can only say that the absence of divalent cations in sea water causes the breakdown of follicles and this is a prerequisite for such artificial spawning.

With respect to the route by which the active substance travels from the radial nerve to the gonad, Chaet (1966b, p. 268) has suggested that "the substance is released from the oral surface of the radial nerve into the sea water, and if not diluted below a threshold level, stimulates the release of gametes from its neighbor." At the same time, "a threshold level of shedding substance would be transported into the coelmic cavity of the original starfish to stimulate its gonad." Although this opinion, that the substance may act as a pheromone rather than hormone, is of interest, the data obtained from the experiment which tested the effect of nerve extract applied from the outside of the body show that the substance is unable to enter the coelomic cavity. In these experiments, one of the doses of nerve extract used (500 ml of sea water containing 200 μg/ml of nerve extract) corresponds to the quantity which is contained in the radial nerves of a single starfish. Since introduction of sea water containing nerve extract at various concentrations into the coelomic cavity through the incision made along the ambulacural plate brought about spawning in almost all the starfish treated, there is no question about the concentration of the nerve extract used. Therefore the phenomenon often observed in the laboratory, that several starfish in the same aquarium begin to shed simultaneously, should not be explained as stimulation of spawning by shedding substances secreted by other starfish. There seem to be some environmental factors, unknown external stimuli, which directly or indirectly induce the release of the active substance from the nerves into the coelomic cavity within each individual.

In regard to the mode of action of the active peptide on induction of spawning and oocyte maturation, recently an intermediate substance was discovered (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967). This second active substance is produced in the ovarian wall under the influence of the active peptide and brings about oocyte maturation as well as spawning. Detailed data as to the production of this substance and its chemical nature will be described in the next paper of this series.

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Summary

1. When a water extract of radial nerves of the starfish, *Asterias amurensis*, was locally applied to one part of an isolated whole ovary for an appropriate period and then small slits were made on the ovarian wall, intense discharge of eggs occurred only in the treated portion.

2. Eggs within the nontreated part of the ovary were observed to adhere to each other and to the gonadal wall by means of the follicle layer surrounding them.

3. Eggs within ligated ovarian fragments which had been treated with nerve extract lost their follicles and underwent maturation. These eggs were found to be freely movable.

4. Artificial spawning could be induced without using nerve extract; ovarian fragments immersed in Mg-free sea water released their eggs after a certain interval, while those treated with Ca-free sea water for an appropriate period spawn after subsequent addition of calcium.

5. Breakdown of follicles occurred within an ovary treated with calcium-free sea water.

6. Treatment with contraction-inducing agents such as potassium chloride and acetylcholine did not bring about spawning, although they caused contraction of isolated ovarian wall preparations.

7. Although the crude nerve extract caused muscular contraction in an isolated rat uterus, gel-filtrated nerve extract failed to induce contraction of either rat uterus or starfish ovarian wall.

8. These findings suggest that the action of the neural substance which is responsible for spawning acts within the ovary to induce breakdown of the follicles surrounding the oocytes.

9. Treatment with hyaluronidase and trypsin failed to induce spawning, suggesting that the intercellular cementing substance between the oocytes resists the action of these enzymes.

10. The neural substance failed to induce spawning when it was applied from the outside of the body.

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